

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**  
**Attorney Docket No. 15344US02**

In the Application of:

Joseph C. Rongione et al.

Serial No.: 10/581,374

Filed: March 12, 2007

For: PRODUCTION AND PURIFICATION OF ESTERS  
OF CONJUGATED LINOLEIC ACIDS

Examiner: Yate Kai Rene Cutliff

Art Unit: 1621

Conf. No.: 3721

**Electronically Filed on  
March 13, 2009**

**AFFIDAVIT OF JOSEPH C. RONGIONE, Ph.D., UNDER 37 CFR §1.132**

I, Joseph C. Rongione, of 53 Second St., Highlands, NJ 07732, declare as follows:

1. I am currently employed by Stepan Company as a Research Associate. I have earned a Ph.D. in organic chemistry from the University of Illinois (1990) and worked in the fine chemical industry for 13 years at the time of the filing of this application. My work in this industry has included the design, synthesis and purification of numerous chemical entities, including working with a variety of distillation units.

2. I am one of the named inventors of patent application Serial No. 10/581,374 entitled "Production and Purification of Esters of Conjugated Linoleic Acids" (the "present application").

3. I have reviewed the non-final Office Action mailed October 15, 2008. I have also reviewed the references cited by the Examiner, and in particular, I have reviewed U.S. Patent No. 6,410,761 ('the Saebo patent').

4. I understand that claims 1-7 of the present application have been rejected under 35 U.S.C. §103(a) as being unpatentable over the Saebo patent. According to the Office Action, Saebo is said to disclose a process of refining conjugated linoleic acid-containing material which utilizes a distillation apparatus to further purify the conjugated linoleic acid-containing material. The Office Action acknowledges that the Saebo patent does not disclose a distillation apparatus that contains a fractionating column, but alleges that including a fractionating column in a distillation apparatus is "routine tweaking" that would be within the purview of one of ordinary skill in the art of using distillation to purify a composition.

5. In 2003, at the time of the priority date of the present application, it was believed that conjugated linoleic methyl esters (CLME) and conjugated linoleic acids (CLAs) were too thermally unstable for distillation with rectification (e.g. utilizing a fractionating column). This belief is illustrated in *Advances in Conjugated Linoleic Acid Research, Volume 2*, Sebedio, J.L., Christie, W.W., Adolf, R., editors, AOCS Press, Champaign, IL (2003). In Chapter 5 of the reference is a discussion of various commercial syntheses of conjugated linoleic acids. Chapter 5 is attached hereto as Exhibit A. Asgier Saebo, the author of Chapter 5 and also one of the named inventors of the Saebo patent, highlights the thermal instability of CLAs at page 73, under the heading "Thermal [1,5] Sigmatropic Rearrangements of CLA Isomers." In particular, Saebo states that the rearrangement of CLA to undesired isomers (e.g., desirable

t10,c2 isomers into undesirable c11,t13 isomers) occurs at 220° C. This reference, which was state of the art at the time of the present application, also recites that molecular distillation should be used to purify the CLA product. (See p. 73 of Chapter 5).

6. The Chapter 5 reference is consistent with the disclosure in the Saebo patent cited in the Office Action. The Saebo patent states, at col. 10, lines 25-35, that distillation of the CLA occurs at 190° C in a molecular distillation plant. According to the patent, the advantage of using molecular distillation is the short time (less than 1 minute) at which the CLA is held at an elevated temperature. The patent further states that conventional batch distillation procedures that involve elevated temperatures of about 180-200° C for up to several hours are to be strictly avoided because of the formation of undesirable isomers. The emphasis on extremely short exposure time and avoidance of high temperature distillation methods shows that Saebo did not believe it would be feasible to introduce rectification (via a fractionating column), with its increased exposure time to elevated temperatures, into the CLA distillation process.

7. In view of the Chapter 5 disclosure that CLAs undergo rearrangement at 220° C, and the Saebo patent's statement that distillation procedures that involve elevated temperatures of 180-200° C for up to several hours are to be strictly avoided for distilling CLA, one would not have expected that a fractionating column, having a heater operating at a temperature of 240° C - 270 ° C, (well above the temperature at which CLA undergoes rearrangement), could even be used in a CLA distillation process. Moreover, given the Saebo patent's disclosure that undesirable isomers form when exposed to elevated temperatures of 180° C - 200° C, one would have expected

an increase in the amount of undesirable isomers in the product stream if a fractionating column were added to the distillation process. Instead, as can be seen from a comparison of Example 1 and Example 6 of the present application, and as further detailed below, use of a fractionating column in the distillation process actually improved both the amount of desirable CLA isomers in the product stream and the product yield. These results were surprising and unexpected given the state of the art, as represented by the Saebo patent and the Chapter 5 reference, in the timeframe of the present application.

8. Example 1 of the present application illustrates a molecular distillation process similar to that suggested by the Saebo patent, utilizing wiped film evaporators (WFE). The Example 1 conditions were as follows:

A CLME stream was distilled in two passes in a wiped film evaporator (WFE). Distillation conditions were: oil temperature range 120-125° C; system pressure 0.05-0.1 mm Hg. The initial CLME stream composition was methyl palmitate (C16:0): 6.20%; methyl stearate (C18:0): 2.37%; methyl oleate (C18:1): 12.65%; methyl linoleate (unconjugated C18:2): 2.42%; CLME (conjugated C18:2): 75.00%. After the two pass distillation run, the CLME stream composition was methyl palmitate: 2.69%; methyl stearate: 2.34%; methyl oleate: 11.37%; methyl linoleate: 2.14; CLME: 80.34%. The fatty acid distribution was determined by GC.

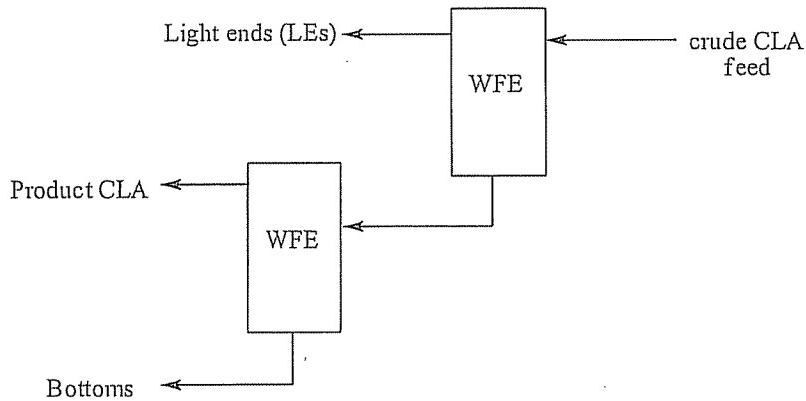


Diagram I. Molecular distillation set up used in example 1.

The overall yield for this apparatus was 60% product, 33% overheads and 7% bottoms.

9. Example 6 of the present application illustrates a distillation process using a rectification (e.g. fractionating) column. The Example 6 conditions were as follows:

A CLME stream was distilled in a unit consisting of a wiped film evaporator (WFE) connected to a rectification column (10 inches of packing). Distillation conditions were: still heater temperature range 240° C - 270° C; system pressure 0.35-0.5 mm Hg (top of the column). Initial CLME stream composition was methyl palmitate: 3.96%; methyl stearate: 2.62%; methyl oleate: 14.57%; methyl linoleate (unconjugated C18:2): 1.00%; CLME (conjugated C18:2): 74.84%. At the end of the distillation run the bottoms stream composition was methyl palmitate: 0.46%; methyl stearate: 2.36%; methyl oleate: 10.56%; methyl linoleate: 0.79%; CLME: 83.03%. The fatty acid distribution was determined by GC.

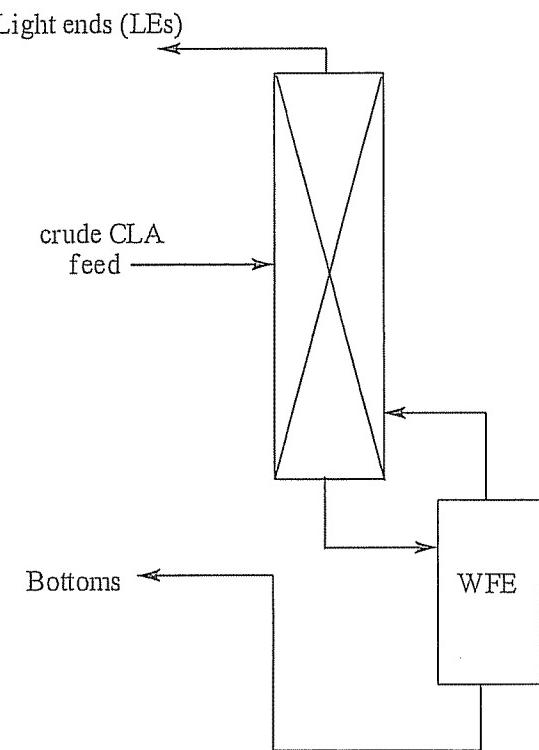


Diagram II. Distillation set up used in example 6.

Using this system gave an improved product yield, 86% product, 10% overheads, and 4% bottoms.

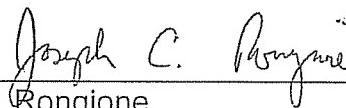
10. Comparing the results of Example 1 and Example 6 shows that the ester stream from Example 1 is clearly inferior to the ester stream recovered in Example 6, although the starting streams were similar. Product CLME was 80.34% in the process of Example 1 and improved to 83.03% in the process of Example 6. This result is surprising since one would expect from the Saebo patent that the increased exposure time to elevated temperatures in the fractionating column in Example 6 would have resulted in a product stream inferior to that of Example 1. Also, the product yield in Example 6 improved by 43%, from 60% in Example 1 to 86% in Example 6, a significant

increase. The improvement in yield along with the improvement in the enrichment of desired isomers shows that the claimed process, utilizing a fractionating column, is a dramatic improvement over the molecular distillation process outlined by Saebo. Taking the various Saebo references to be the standard state of the art in the timeframe of the present application, the introduction of a fractionating column into the distillation of CLA streams provides an unexpected result. The addition of a fractionating column to a wiped-film evaporator cannot be viewed as process tweaking since Saebo rejected such distillation procedures involving increased exposure time to elevated temperatures. ('761, column 10, lines 29-36).

11. I declare that all statements made herein to my knowledge are true and that all statements made on information and belief are believed to be true and, further, that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under 18 U.S.C. §1001, and that such willful false statements may jeopardize the validity of this application and any patent issuing thereon.

Dated: March 13, 2009

Respectfully submitted,

  
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Joseph C. Rongione

# EXHIBIT A

## Chapter 5

# Commercial Synthesis of Conjugated Linoleate

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## Introduction

Conjugated linoleic acid (CLA) has been available as a health food supplement in soft gelatine capsules since 1995 in the United States, and more recently in several European countries and Japan. CLA products designed for food and animal feed additives are expected to appear on the market in the near future. CLA has been produced for decades for technical purposes and continues to be used as a substitute for Chinese tung oil in the paint and varnish industry due to its "drying" characteristics. The production methods developed for technical CLA products were rapidly modified and improved upon after the discovery of the biological activity of the substance. This chapter will focus on supplements in particular, including current production methods, stability, and breakdown products. Purified isomers are currently available only for research purposes, but a few references to methods available for purification will be given.

## CLA for Technical Applications

### *Dehydration of Ricinoleic Acid*

Several decades ago, only two natural oils (tung oil and oiticica) were known to contain conjugated double bonds. Oils that contain these bonds rapidly form a polymer film ("drying") if a thin layer is exposed to air; tung oil was widely used in the paint and varnish industry. An increasing demand for such oils and limited availability encouraged efforts to produce drying oils from nonconjugated oils.

The main constituent of castor bean oil is ricinoleic acid (12-hydroxy-9-octadecenoic acid). Around 1937, dehydrated castor oil appeared on the market in the United States as a substitute for tung oil. Ten years later the product was established as one of the most popular drying oils (1). It has been known since 1888 that castor oil could be dehydrated, and since 1914 it was known that the main isomers of linoleic acid formed had double bonds at positions 9,11 and 9,12, but the detailed composition of dehydrated ricinoleic acid was not investigated until recently. A German patent from 1930 (2) and a U.S. patent from 1934 (3) describe the preparation of dehydrated castor bean oils. A modified procedure was recently used to produce an 83% pure 9-*cis*,11-*trans* CLA concentrate from purified ricinoleic acid (4). Main impurities were the 9-*cis*,11-*cis* and 9-*cis*,12-*trans*-octadecadienoic acids. Conventional dehydration

using high temperatures will create other isomers, mainly 8-*trans*,10-*cis* and *trans*,*trans* isomers. CLA from dehydrated castor oil is not available on the market in supplement form. Apart from safety issues, the reason is the absence of 10-*trans*,12-*cis* CLA, the isomer shown to inhibit fat synthesis (5).

### ***Alkali Isomerization of Linoleic Acid Oils***

Attempts to produce drying oil from nonconjugated oils were successful in the late 1930s as well as for oils containing methylene-interrupted fatty acids. In 1941, a U.S. patent was issued that describes the use of monohydric and polyhydric alcohols as solvents and a variety of alkaline catalysts (6). A few years later, two patents were issued that described the use of water (7) and steam (8), respectively, as solvent in an autoclave to achieve the temperatures necessary to conjugate unsaturated acids. It is actually the soap that is conjugated. Upon addition of mineral acid, the conjugated free fatty acids are liberated. Currently, CLA is produced for technical purposes in high alkaline water at ~230°C. Feedstock is usually free fatty acids (after fat splitting to recover glycerol). The product is usually distilled to yield a virtually colorless oil.

## **Production of CLA for Animal and Human Consumption**

### ***Alkaline Water Isomerization***

The first products to appear on the health food market contained ~65% CLA, and the profile of the CLA isomers was similar to technical-grade products. Christie *et al.* (9), showed that the main isomers of CLA in addition to 9-*cis*,11-*trans* and 10-*trans*,12-*cis* were an 8,10 and an 11,13 isomer *cis,trans* or *trans,cis*. These were later identified as 8-*trans*,10-*cis* and 11-*cis*,13-*trans* (10). Such products are still available as supplements, and most if not all are produced from linoleate-rich starting materials in high-alkaline water reactions at temperatures >230°C. We investigated reaction parameters in water alkaline (KOH or NaOH catalyst) reactions trying to avoid formation of 11-*cis*,13-*trans* and 8-*trans*,10-*cis*. It turned out not to be possible to achieve a nearly quantitative isomerization and at the same time avoid formation of the said isomers (data not published).

### ***Isomerization in Propylene Glycol***

Quantitative isomerization of oils containing polyunsaturated fatty acids in monohydric and polyhydric alcohols was described in 1941 (6). A detailed procedure using ethylene glycol is described in a patent from 1996 (11). Ethylene glycol has not been used commercially for production of CLA for consumer safety reasons. Propylene glycol has therefore been selected by several producers who independently developed proprietary procedures (12,13). KOH was selected as catalyst because of its high solubility compared with NaOH. Reaction temperatures are typically 130–180°C, and times of reaction are from 3 to >24 h. The quantity of KOH

is substantial and in excess of that needed for quantitative saponification. After the reaction is complete, the mixture is cooled down and water and mineral acid (hydrochloric or sulfuric) are added. Free fatty acids of CLA are liberated as soon as the mixture becomes acidic. One patent describes the use of hexane at this point to extract CLA and facilitate separation from the bottom aqueous layer without emulsion problems. However, the operation is possible without the use of hexane. For the sake of recovery of propylene glycol, free fatty acids are preferred as feedstock oil. A triacylglycerol feedstock will create glycerol to contaminate the propylene glycol. After water and solvent (hexane if used) have been removed under vacuum, the CLA product is preferably purified by deodorization and distillation. Peroxides and volatiles are easily removed by deodorization. The peroxides are broken down to secondary volatile products that are removed in the process.

The purification process should also include a molecular distillation step to remove nonvolatile compounds such as polymers, sterols, and propylene glycol esters. Heavy metals could also arise from the isomerization process if mineral acids are used in stainless steel reactors (14). Their concentrations are reduced upon molecular distillation as well. A distilled product is almost colorless and has an acid value of ~200 (mg KOH/g). A nondistilled product might have an acid value of ~190, be yellow to slightly brown in color and have an opaque appearance. However, we have observed a slight decrease in acid value in capsules over time and also a darkening of the oil if the capsule material is colored. Due to the strong alkaline process, free fatty acids are the final product regardless of the form of feedstock (free fatty acid, a monoalkyl ester, or a triacylglycerol oil). Therefore, CLA in supplements are offered almost exclusively as free acids, in contrast to n-3 concentrates that are offered either as ethyl esters or reesterified triacylglycerols.

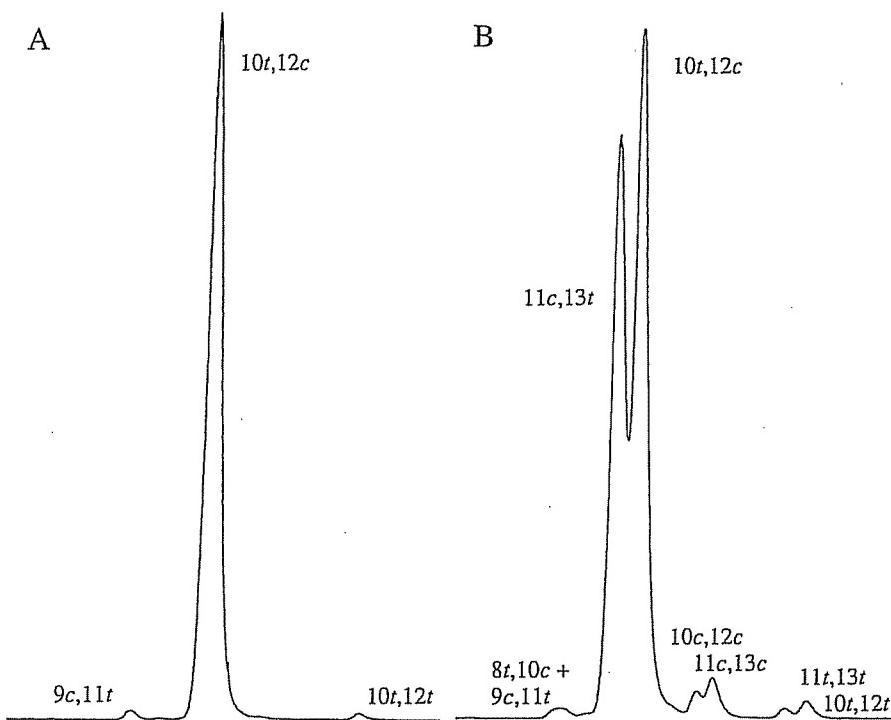
#### *Isomerization of Mono-Alkyl Esters Using Alkali Metal Alcohohlates*

Recently, a proprietary method has been developed that quantitatively isomerizes methyl esters and ethyl esters of linoleic acid using very low quantities of catalysts and virtually no solvents (data not published). Because of the quantity of catalyst (~2%), only a small fraction of the ester is saponified and hence appears as free fatty acid after addition of a neutralizing agent. Most of the product (>92%) is still in the form of the methyl or ethyl ester after the isomerization process. The reaction proceeds at temperatures down to below 100°C, and the CLA product is characterized by very low levels of CLA isomers produced by thermal [1,5] sigmatropic rearrangements (see below).

#### *Thermal [1,5] Sigmatropic Rearrangements of CLA Isomers*

Production of CLA in propylene glycol or other alcohol under mild conditions gives rise to <0.5% each of the isomers 11-cis,13-trans and 8-trans,10-cis. After purification of single isomers, we showed that upon heating to 220°C in an inert atmosphere, 10-trans,12-cis gives rise to 11-cis,13-trans (Fig. 5.1). Upon heating an 11-cis,13-trans concentrate, 10-trans,12-cis was produced. Under optimal condi-

tions, an equilibrium is established between these isomers, and only minor quantities of *cis,cis* and *trans,trans* isomers are formed. The isomer shift is actually a thermal [1,5] sigmatropic rearrangement, (Fig. 5.2) allowed according to the orbital symmetry theory (Woodward-Hoffmann). For this sigmatropic rearrangement to occur, it is essential that one of the bonds be in the *cis*-configuration. A similar rearrangement is observed for the isomers 9-*cis*,11-*trans* and 8-*trans*,10-*cis*. The phenomenon is actually a tool for chemists to produce new isomers. Any given CLA isomer that contains one double bond in the *cis*-configuration and one in the *trans*-configuration can be heated to be isomerized into another specific *cis,trans* or *trans,cis* isomer. Isomers formed might be predicted from formulae as in Fig. 5.2. A simple rule of thumb is that the two double bonds will move against the *cis* end of the bond pairs. For example, 7-*trans*,9-*cis* (a common isomer in milk fat) will isomerize to 8-*cis*,10-*trans* and vice versa. Prolonged heating of isomers



**Fig. 5.1.** Gas chromatography (GC) profile of ethyl ester of purified 10-*trans*,12-*cis* CLA isomer (a) before and (b) after heating to 220°C in an inert atmosphere for 2 h. The process caused isomerization into the isomer 11-*cis*,13-*trans* by thermal [1,5] sigmatropic hydrogen shift. GC conditions: 100-m CP Sil 88 fused silica capillary column and flame ionization detection (FID).

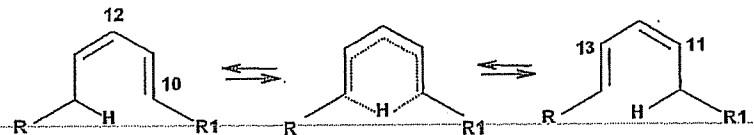


Fig. 5.2. Drawing explaining thermal [1,5] sigmatropic rearrangement between the CLA isomers 10-*trans*,12-*cis* and 11-*cis*,13-*trans*. Reaction is spontaneous and the transition state depicted in the middle is not an intermediate product. R=  $-(CH_2)_4$  and R<sub>1</sub>=  $-(CH_2)_8CO_2H$ .

seems to gradually develop *cis,cis* and *trans,trans* isomers. Impurities present (iron, copper and other metals) will greatly favor formation of *trans,trans* isomers.

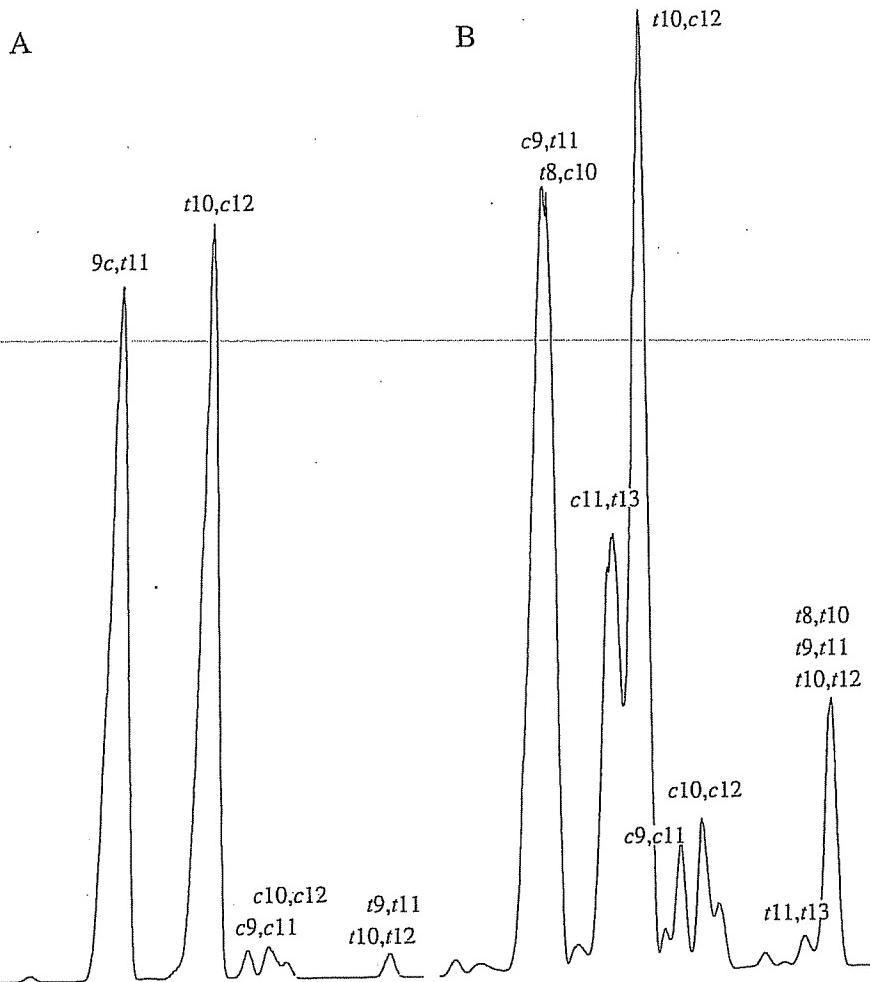
#### **Isomer Profile in Available Supplements**

The total content of CLA in supplements more or less reflects the starting material. Sunflower oil as a starting material results in ~65% CLA, whereas safflower oil yields up to 80%. Both oils contain a level of palmitic acid that tends to cause precipitation below room temperature. Products are now available with a reduced content of saturated acids and >80% CLA. The products can be classified in two groups, the “4-isomer product” and the “2-isomer product” (Fig. 5.3). The latter product contains almost exclusively 9-*cis*,11-*trans* and 10-*trans*,12-*cis*, both up to ~38% of the oil, or almost 50% of the CLA. The former, however, contains several isomers. The elution order on gas chromatography (GC) of the 4 main peaks is 9-*cis*,11-*trans*; 8-*trans*,10-*cis* (may co-elute with 9-*cis*,11-*trans*); 11-*cis*,13-*trans*; and 10-*trans*,12-*cis* (9). In addition a major *trans,trans* peak (9,11 and 10,12 co-eluting) often reaches the same level. Such products may contain as little as 8% 10-*trans*,12-*cis*. Despite co-elution, the content of 8-*trans*,10-*cis* can be estimated approximately by measurement of 11-*cis*,13-*trans*. Both are produced to the same degree from their mother components. In other words, the ratio of 11-*cis*,13-*trans* to 11-*cis*,13-*trans* + 10-*trans*,12-*cis* equals that of 8-*trans*,10-*cis* to the co-eluting peak 8-*trans*,10-*cis* + 9-*cis*,11-*trans* (data not published). Products from a single source have been reported to show substantial variation in isomer profile (15), and products also are available that contains virtually no (present data, Table 5.1) or totally lack CLA (10). Two of 17 products sampled and analyzed in January–March 2002 by our laboratory contained high levels of the isomers 11-*cis*,13-*trans* and 8-*trans*,10-*cis* (Table 5.1).

#### **Stability and Breakdown Products of CLA Preparations**

##### **Stability of CLA Compared with Linoleic Acid**

A few studies report data on the stability of CLA compared with linoleic acid in different test models. Bubbling of oxygen through samples at 90°C resulted in a



**Fig. 5.3.** Partial gas chromatography (GC) profile of ethyl esters of (a) a "2 isomer type" and (b) a "4 isomer type" CLA supplement, using a 100-m CP Sil 88 fused silica capillary column and flame ionization detection (FID). Product (a) is identical to product No. 14 and product (b) is identical to No. 17 in Table 5.1. Note co-elution of 8-trans,10-cis and 9-cis,11-trans.

much higher peroxide value (PV) in linoleic acid (16) than for CLA. When a mixture of CLA isomers was heated to 50°C in air, the rate of oxidation was considerably faster for CLA than for linoleic acid. The rate of oxidation was measured as "remaining CLA" by GC. When comparing groups of CLA isomers, stability decreased in order of *trans,trans* > *cis,trans* or *trans,cis* > *cis,cis*. (17). In a study in aqueous and solvent systems measuring stability by the induction period system,

**TABLE 5.1**

Content of CLA (% of Total) in 17 Commercial Supplements Sampled in January–March 2002<sup>a</sup>

Product	Product type	Country	%CLA	%10t,12c	%11c,13t	Acid value
1	Soft gelatine capsule	Norway	80.1	47.8	0.4	197
2	Liquid	Norway	78.6	47.1	1.8	2
3	Soft gelatine capsule	Norway	69.1	46.7	1.2	196
4	Soft gelatine capsule	Norway	78.3	48.7	0.3	197
5	Soft gelatine capsule	Norway	76.4	46.6	1.3	193
6	Soft gelatine capsule	U.S.	71.4	46.3	0.5	189
7	Soft gelatine capsule	U.S.	74.8	43.1	0.9	192
8	Soft gelatine capsule	U.S.	77.9	48.5	0.3	199
9	Soft gelatine capsule	U.S.	70.8	44.4	0.6	189
10	Soft gelatine capsule	U.S.	79.6	45.3	0.4	193
11	Soft gelatine capsule	U.S.	72.0	44.4	2.3	192
12	Soft gelatine capsule	U.S.	74.3	43.6	1.0	187
13	Soft gelatine capsule	U.S.	61.5	28.5	0.8	180
14	Soft gelatine capsule	U.S.	76.3	48.4	0.3	196
15	Liquid, emulsion	U.S.	1.2	47.8	0.3	NA
16	Soft gelatine capsule	S. Africa	51.7	16.5	16.1	198
17	Soft gelatine capsule	Norway	57.7	29.9	16.5	200

<sup>a</sup>The isomers 10-*trans*,12-*cis* and 11-*cis*,13-*trans* are expressed as the percentage of total CLA. Only two products were of the "4 isomer" type. Two products were liquids, one oil and one emulsion (1.7% fat). Content of 9-*cis*,11-*trans* (not tabulated due to overlap with 8-*trans*,10-*cis*) is approximately equal or slightly less than 10-*trans*,12-*cis* in all supplements currently available. Distilled products typically have acid values of 195–200 mg KOH/g. (A 100.00% free fatty acid product of oleic acid has a theoretical acid value of 198.60). CLA region of product 14 and product 17 is illustrated in Figure 5.3. NA, not available.

CLA was more stable than linoleic acid as free fatty acids, and less stable as ethyl esters (18). Another study using methyl esters reported that stability decreased in the following order: oleate > CLA > linoleate. Samples were stored in the dark at 40°C and monitored by thin-layer chromatography (TLC), GC and PV. From 9-*cis*,11-*trans*, the major monohydroperoxides formed were identified as 8-, 9-, 12- and 13-monohydroperoxides, whereas 10-*trans*,12-*cis* yielded primarily 9-, 10-, 13-, and 14-monohydroperoxides (19).

Data reported on the PV of CLA preparations are consistent with our observations. CLA do not easily develop high PV, yet the oxidative breakdown of CLA seems comparable to that of linoleic acid. The reason is likely to be a more rapid breakdown of peroxides into secondary oxidation products.

#### Volatiles

In a pilot project on developing a procedure for CLA production, a high content of hexane was observed in a product by headspace GC-mass spectrometry. After searching for the source of contamination, it was finally concluded that pentane

and hexane are among the secondary oxidation products of CLA. This was later confirmed by experiments. To our knowledge, hexane has never been reported to be an important inherent oxidation product of vegetable oils. In a free fatty acid concentrate of 9-*cis*,11-*trans* stored in the dark with air access for 1 wk, the two major volatiles that developed were, not surprisingly, heptanal and 2-nonenal. The concentration increased from 4.8 and 0.7 to 84.6 and 22.5 µg/g, respectively. Volatile breakdown products seem not to build up in soft gelatine capsule supplements. A CLA product that was stored for 5 y at room temperature contained 2.3 µg/g hexanal and 2.2 µg/g heptanal (data not published). No antioxidant was added to the supplement.

Among less volatile breakdown products, furan fatty acids were reported when air was bubbled through CLA dissolved in a mixture of methanol and water at 50°C. (20). Furanoid fatty acids might also arise in preparation of fatty acid methyl esters (FAME) for GC. To our knowledge, furan fatty acids have not been reported as an oxidative breakdown product in dry oil preparations of CLA.

### *Polymers*

Conjugated oils are considered valuable raw materials for the paint and varnish industry because of their film forming properties ("drying") upon air access. This property gives rise to concern regarding the stability of CLA preparations. In a stability test program, 10 mL of CLA triacylglycerols and free fatty acids were stored in an amber open glass bottle in darkness. After 4 mo at 25°C, controls without antioxidants added were highly viscous and not suitable for further stability testing. The samples had a membrane layer on the surface, and the viscosity clearly developed over time. Samples with antioxidants did show a retarded viscosity development (data not published).

Soft gelatine capsules are considered to give reasonable protection from exposure of unsaturated oils to air. Capsules containing CLA free fatty acids showed a slight increase in polymer content from 1% in freshly prepared capsules to 7% after 5 y (data not published). For comparison of health risks, a limit for rejection on cooking oils has been established in some countries; values listed in a report from the European Parliament are 16% (Holland), and 10% (Belgium and Czech Republic) (21).

### *Stability of CLA in Soft Gelatine Capsules*

No data have yet been published on the stability of CLA in capsules. Observations on polymers and volatiles in capsules are reported above. In a stability test program according to International Conference on Harmonization (ICH) guidelines on a free fatty acid product, the content of total CLA was not significantly reduced after 24 mo at 25°C/60% relative humidity. In this test, CLA was measured by GC. Peroxide value (PV) did not develop in the capsules (data not published).

## Next Generation Products

### *Isomer Purification*

All CLA supplements currently offered contain approximately equal amounts of 9-*cis*,11-*trans* and 10-*trans*,12-*cis*. The extra costs of producing a biased isomer product might be justified if beneficial health effects were documented. The 9-*cis*,11-*trans* and the 10-*trans*,12-*cis* isomers of CLA are now available for research purposes in kilogram scale with a purity of ~90%. In small quantities, purities up to 99% are offered. High yields and high purity can be obtained by repeated crystallization of the methyl ester forms in acetone at temperatures as low as -60°C (22).

A concentrate with 83% 9-*cis*,11-*trans* isomer was obtained from gentle dehydration of ricinoleic acid from castor bean oil and subsequent purification steps (4). The use of urea inclusion compounds does not seem to be a feasible procedure to separate 9-*cis*,11-*trans* and 10-*trans*,12-*cis* (23). Enzymes, however, are promising tools for these separations. A 98% concentrate of 9-*cis*,11-*trans* was reported by using lipase from *Geotrichum candidum*. The enzyme was capable of esterifying selectively 9-*cis*,11-*trans* to monohydric alcohols from a mixture of several isomers (24). A patent has been issued on purification and characterization of isomerase from *Propionibacterium acnes* and *Clostridium sporogenes*. The purified isomerase preparations were able to quantitatively isomerize linoleic acid into the 10-*trans*,12-*cis* isomer of CLA (25).

### *Triacylglycerols for Food Applications*

Free fatty acids and monoalkyl esters are applicable to supplement capsules and probably also to animal feed formulations. However, as an ingredient in food for human consumption, CLA is most attractive as a triacylglycerol. A nonspecific lipase has been reported to esterify CLA with glycerol very efficiently (26). Incorporation of CLA into food fats and oils has also been reported for fish oils (27), butterfat (28,29), and corn oil (30). A bottled triacylglycerol product, stabilized with antioxidants, has been available in the health food market in Scandinavia since 2000. Flavor and antioxidants are added to the oil designed to be taken by spoon. Further technical developments of CLA products improving the stability and applicability as well as addressing specific issues of food legislation will require attention before CLA can be made available as an ingredient for animal feed and human food.

## Summary

CLA supplements for human consumption have been available since 1995, and most of the products contain between 60 and 80% CLA in the form of free fatty acids. The history of CLA produced for technical purposes dates back almost 100

y, however. The isomer profile of the supplements range from an almost pure 9-*cis*,11-*trans* + 10-*trans*,12-*cis*-50/50 mixture (made in alcohol solvents between 100 and 150°C), to a mixture with four prominent *cis,trans* or *trans,cis* isomers produced in high alkaline water at high temperatures, of which 8-*trans*,10-*cis* and 11-*cis*,13-*trans*-18:2 are produced from 9-*cis*,11-*trans* and 10-*trans*,12-*cis*, respectively, by thermal [1,5] sigmatropic rearrangements of the isomers. Supplements are typically offered as free fatty acids in soft gelatine capsules. Unpublished data on stability of CLA-in-capsules-stored-according-to-ICH guidelines for 2 y did not show any loss of active ingredient.

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# **Advances in Conjugated Linoleic Acid Research, Volume 2**

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